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Evaluation of the Consuming Zinc (Zn) and Iron (Fe) Elements as the Immune System Boosters in MCF-7 Cell Culture Media Treated by Pomegranate Parenchyma and Turmeric Extracts

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Abstract

Essential minerals and trace elements have well-characterized physiological functions within the body and must be supplied by the diet.

The aim of this study is an analytical model for consuming the trace elements (Zn and Fe) in MCF-7 cell culture media in three different times and comparing.

This study was undertaken in two parts: First, the cell cultures of MCF-7 and MTT, aiming at determining cell viability, were reported. Second, the elements of Zinc and Iron by Atomic Absorption Spectrophotometry. All the data were analysed by SPSS version 23, ANOVA test. Results are expressed as the mean ± SEM.

In cell viability test the highest rate of decrease was observed in group C; Further, increase highest rate were in groups A in two timeframes. In Zinc samples, the only increase had been observed in group A and decrease highest rate was shown in group A. In Iron samples, decrease highest rate was shown in group E. Further, the highest rate of increase was observed in group A.

For conclusion of all the tests, in control groups, we had cell growth and the amount of Iron increasing and Zinc decreasing. But for the other tests, in terms of non-combinations all the tests are descending (Except viability for group B). The dominant effective material of turmeric is Curcumin have phenolic ring with high affinity. So, it may need a catalyser for best effect. So, more study with combination with other dosages to matching the herbals are suggested.

Keywords: MCF-7 cell line; Pomegranate; Turmeric; Zinc; Iron; Cell viability

Abbreviations

ETE: Ethanolic Turmeric Extract; MPPE: Methanolic Pomegranate Parenchyma Extract; MCF-7: Michigan Cancer Foundation-7; MTT: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; Zn: Zinc; Fe: Iron; ELISA: Enzyme Linked Immuno Sorbent Assay; DMSO: Dimethyl sulfoxide; RPMI: Roswell Park Memorial Institute (RPMI) 1640 medium; PBS: Phosphate Buffer Saline; Penstrep: Penicillin + Streptomycin.

Introduction

Breast cancer is considered a global public health problem due to its increasing incidence and associated socioeconomic implications. It is the most frequent cancer among women in the world. Breast cancer is a complex multifactorial disease in which genetic and environmental factors are involved [1]. Essential minerals and trace elements have well-characterized physiological functions within the body and must be supplied by the diet.

Zinc is essential even in the earliest stages of an immune response [2]. Zinc is used for cell growth and is also useful in maintaining the integrity of cell membranes, therefore, cancer cells may absorb zinc from the circulation to maintain cancer growth and membrane integrity [3,4].

Iron plays an essential role in Immunosurveillance, because of its growth promoting and differentiation-inducing properties for immune cells and its interference with cell-mediated immune effector pathways and cytokines activities [5].

MCF-7 is a mammary gland cell which is derived from metastatic site of pleural effusion of an adenocarcinoma, 69-year adult woman. Cell is adherent in cell culture media by chromosome modal number of 82 (range 66-87). The stem line chromosome numbers ranged from hypotriploidy to heteroploidy, with the 2S component occurring at 1%(ATCC ® HTB-22™).

Pomegranate (Punica granatum L) is a grate natural source of Phenolic components containing antioxidants such as Tannins, Polyphenols, Flavonoid and Vitamin C. These herbal biochemical properties in cancerous cells are survivor for restoration properties [6-8]. Pomegranate fruit extraction inhibits the skin cancer because of inhibition of MAP Kinase in epidermal keratinocytes [9].

Turmeric (Curcuma longa L) is a rich source of phenolic compounds, namely, curcuminoids. Turmeric is herbaceous perennial plant belonging to the botanical family of Zingiberaceae, or the ginger family [10]. Curcumin can alter the expression of genes involved in the cancer signaling pathway to affect and inhibit cancer initiation,

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promotion, tumor cell survival and proliferation, angiogenesis, and metastasis stages [11].

Methodology

This study considered into two parts: First the cell culture of MCF-7 and MTT to determine cell viability and second, to measure the elements of Zinc and Iron by Atomic absorption. All the test performed in 3 timeframes of 24, 48 and 72 hours after extracts additions.

Ethanolic Turmeric Extraction (ETE)

Ethanolic Turmeric Extraction (ETE)5.2 Methanolic Pomegranate Parenchyma Extraction (MPPE)

Each 100 gms of pomegranate provided from sourmagh was sieved 3 times and the powder was mixed with 400 mL of methanol 90%. After 48 hours the liquid was passed through a number 40 sieve papers, the scum was then mixed with methanol 70% and again sieved; after 24 h these two solutions were mixed. After distillation stage by evaporator, using a freeze dryer the extraction was dried and changed to powder. 3-4 grams of pure extraction of pomegranate was prepared [13].

Cell culture

At the present study, MCF-7 cells were purchased from Iran Pasteur Institute and cultured. Later, the viability of cells was measured by MTT method. Finally, the rate of Zn and Fe were measured through atomic absorption has been performed [14-16].

MCF-7 cells were after being removed from nitrogen tank and warming up, centrifuged by 200 gms and cultured in 25 ml flasks containing media. The media was DMEM which was filled with 10% solution of Fetal Bovine Serum (FBS) and 1% Penicillin and Streptomycin (Penstrep) made of Sigma-Aldrich. Cell culture was performed in 6 groups as follows:

Group A: Control: Only 0.1 milliliter of ethanol 50%+formic acid 2% were added to media.

Group B: ETE 100%

Group C: MPPE 100%

Group D: ETE 25%+MPPE 75%

Group E: ETE 50%+MPPE 50%

Group F: ETE 75%+MPPE 25%

In the present study, the 50 μM ETE dosage was used for group B and determined the base of 100% for ETE. Further, the authors used the 25%, 50% and 75% of this amount for the other groups as mentioned below.

The dosage of 250 mg/ml was chosen for group C and formed the base of 100% for MPPE. In the present study, the authors used the 25%, 50% and 75% of this amount for other groups as mentioned below.

Cell viability test

This method is based on succinate dehydrogenase activation in live cell mitochondria that changes yellow color MTT solution into purple color Formosan. After reaction, it could be measured by ELISA reader after addition of DMSO.

Methods

2 ml RPMI media and the cells from each cell culture plate were added into ELISA wells. After 24 hours incubation, 20 μL prepared MTT dye was added into each well (The procedure through which MTT dye was prepared was as follows: 5 mg MTT dye powder in 1ml PBS). Then, the plate was incubated for 3 hours. The plate content was wasted and 200 μL DMSO was added into each well. The plate was measured by ELISA reader at 560 nm wavelength [17].

Analysis methods

The Zinc (Zn) and Iron (Fe) has been measured in extractions derived from culture media and cells.

To measure trace elements (Zn and Fe), 0.5 mL of extracts and 0.5 mL of digesting solution (nitric acid: percholeric acid (70:30)) were mixed and sera were digested at 90˚ C-100˚ C in water bath for 16-20 hours. Then the volume of the samples was increased to obtain primarily volume (1 mL), by distilled water. The zinc and iron were measured in AIR-C2H2 (Flame) by Atomic absorption spectrophotometry [18].

Statistical analysis

The statistical analysis was performed using ANOVA followed by the Duncan test. The results were expressed as the mean ± SEM. Further, a P-Value of 0.05 or below was considered as the criterion to determine the significance of the statistical test.

Results

The cell viability results revealed significant differences between the treatment and the control groups as shown in Table 1 below.

In timeframe 24 hours, there were significant differences between all groups and control except group B and the control group ($p<0.05$). All groups in time frame 24 hours revealed significant differences with each other except two group pairs namely E and C as well as F and B ($p<0.05$). In the same vein, in timeframe 48 h, significant differences were observed between the control and the treatment groups except group B ($p<0.05$) [19]. Finally, in timeframe 72 hours, the analyses revealed significant differences between all the treatment groups and the control one. The highest rate of decrease in MTT absorbance was observed in group C in timeframes of 48 hours to 24 hours ($p<0.05$). Similarly, the highest rate of increase in MTT absorbance was observed in group A between 2 timeframes of 48 hours and 24 hours (48 to 24) and also 2 timeframes of 72 hours and 48 hours (72 to 48) with the same percentage of c, f in Table 1.

The Zinc samples also have been measured in 3 timeframes of 24, 48 and 72 hours after treatment with ETE and MPPE of c, f in Table 2 .

At the present study, there were significant differences between all groups in timeframe 24 hours ($p<0.05$). In timeframe 48 hours significant differences between all groups were observed except group B and E. Further, all groups were revealed significant differences with group A ($p<0.05$). Significant differences have been observed in timeframe 72 hours between all groups and control except D and E ($p<0.05$) [20]. In group A, there were significant differences between all timeframes ($p<0.05$). Further, in all groups there were significant differences between all 3 timeframes ($p<0.05$). In all Zinc samples, the only increase was observed in control in timeframe 48 hours to 24 hours. At the present study, the highest rate of decrease was shown in group A in timeframe 72 hours to 48 hours of c.f in Table 2.

The Iron samples also have been measured in 3 timeframes of 24, 48 and 72 hours after treatment with ETE and MPPE of c.f in (Table 3).

Groups	Control (A)	ETE 100% (B)	MPPE 100% (C)	ETE 25% + MPPE 75% (D)	ETE 50% + MPPE 50% (E)	ETE 75% + MPPE 25% (F)
24 hours	0.6274 ± 0.0169	0.5578 ± 0.0191	0.2536 ± 0.1491	0.1222 ± 0.0140	0.3220 ± 0.0107	0.4806 ± 0.0080
48 hours	0.8282 ± 0.0143	0.7224 ± 0.0160	0.0996 ± 0.0049	0.1376 ± 0.0035	0.2550 ± 0.0129	0.5864 ± 0.0206
72 hours	1.1010 ± 0.0401	0.9532 ± 0.0201	0.0978 ± 0.0035	0.1154 ± 0.0066	0.2122 ± 0.0106	0.7252 ± 0.0226

Table 1: Cell viability of under treatment and control groups in 24, 48 and 72 hours after extract addition.

Groups	Control (A)	ETE 100% (B)	MPPE 100% (C)	ETE 25% + MPPE 75% (D)	ETE 50% + MPPE 50% (E)	ETE 75% + MPPE 25% (F)
24 hours	0.2806 ± 0.0028	0.2696 ± 0.0006	0.2618 ± 0.0007	0.2858 ± 0.0018	0.2970 ± 0.0016	0.2548 ± 0.0005
48 hours	0.2908 ± 0.0010	0.2572 ± 0.0003	0.2498 ± 0.0008	0.2638 ± 0.0009	0.2594 ± 0.0012	0.2430 ± 0.0007
72 hours	0.2506 ± 0.0020	0.2466 ± 0.0006	0.2392 ± 0.0008	0.2532 ± 0.0010	0.2490 ± 0.0007	0.2326 ± 0.0007

Table 2: Zn in 3 timeframes of 24, 48 and 72 hours after ETE and MPPE treatment.

Groups	Control (A)	ETE 100% (B)	MPPE 100% (C)	ETE 25% + MPPE 75% (D)	ETE 50% + MPPE 50% (E)	ETE 75% + MPPE 25% (F)
24 hours	0.3426 ± 0.0040	0.3824 ± 0.0021	0.3854 ± 0.0027	0.4018 ± 0.0019	0.5518 ± 0.0064	0.3262 ± 0.0027
48 hours	0.5010 ± 0.0047	0.4110 ± 0.0021	0.3478 ± 0.0024	0.3058 ± 0.0017	0.3070 ± 0.0022	0.2958 ± 0.0028
72 hours	0.3530 ± 0.0024	0.3152 ± 0.0027	0.3290 ± 0.0015	0.3334 ± 0.0027	0.3056 ± 0.0009	0.3314 ± 0.0025

Table 3: Fe in 3 timeframes: 24, 48 and 72 hours after ETE and MPPE treatment.

All groups revealed significant differences comparing with group A ($p<0.05$). In timeframe 24 hours, there were observed significant differences between all groups except groups B and C ($p<0.05$). In timeframe 48 hours, except groups D and E, the rest of groups showed significant differences with each other ($p<0.05$). At the present study, the highest rate of decrease was observed in group E between timeframes of 48 hours to 24 hours. The highest rate of increase was observed in group A in timeframes 48 hours to 24 hours respectively in c.f in Table 3.

Discussion

The conclusion of the present study was the eradication of cancerous cells by using a mixture of ethanolic turmeric extract and methanolic pomegranate parenchyma extract, in MCF-7 cell culture media, by boosting the cellular immune system. This capability is rooted in the trace elements that are present in the extracts. In a study in Iran at 2010 (1389 Iranian calendar) posited that in bladder cancer patients the amount of Zinc and Iron is less than healthy people and copper is more than them and accordingly advised that more studies about the ratio of Zinc and Copper in cancer patient in a study by in Iran, the results explained that comparing copper and the ratio of copper to zinc in benign and malignant ovary cancer patient showed an increasing in serum level in malignant cancer patients. Shobeiri suggested that this comparison can be a method for diagnosis of disease.

At the present study, cell viability determination was performed before and after extracts addition. In chart the highest rate of decrease in MTT absorbance were observed in Group C in timeframes of 48 hours to 24 hours in amount of 60.7% in Figure 1. It reveals that the most effective dosage on cell culture in this part is MPPE 100% (in timeframe 72 hours to 48 hours we had not anymore viable cell). In a study posited that curcumin and nano-curcumin causes cell proliferation to reduce in colon cancer; but this reduction was greater in nano-curcumin group. In a study in 2014 by result showed a decreasing trend in cell viability with increasing concentrations of curcumin and ETE. A higher response occurred with curcumin 75 μM.

The least rate of decrease in MTT absorbance, 1.8%, was observed in group C in timeframes of 72 hours to 48 hours shown in Figure 1. In a study in Iran showed the cytotoxic effects of pomegranate seed extracts on MCF-7. In another study in Iran by the authors concluded that extract of black pomegranate parenchyma could induce apoptosis, cell death and morphological change of Melanoma, but it had no side effect on endothelial cells. They suggested that it could be used as a suitable treatment aid for apoptosis induction in melanoma patients having passed complemented tests.

In the present study, we had pure ETE, pure MPPE and 3 combinations of ETE and MPPE treatments. The least effective dosage was ETE 100% of group B and the most effective dosage was a combination of ETE 25% and MPPE 75% of group D. It means that the highest rate of mortality is observed in treating MCF-7 by a combination of ETE and MPPE in 1: 3 dosages, respectively. The highest rate of MTT absorbance increase was seen in group A (Control) between 2 timeframes of 48 hours and 24 hours (48 to 24) and also 2 timeframes of 72 hours and 48 hours (72 to 48), in the same percentage of 32% shown in Figure 1. This finding shows that cells were growing without any problems. This amount was very close to the amount of group B (ETE 100%) in timeframe of 72 hours to 48 hours that was 31.9% shown in Figure 1. The DNA microarray analysis revealed that pomegranate extract downregulated genes associated with mitosis, chromosome organization, RNA processing, DNA replication and DNA repair, and upregulated genes involved in regulation of apoptosis and cell proliferation in both microarray and quantitative RT-PCR indicated that pomegranate extract downregulated important genes involved in DNA double strand break (DSB) repair by homologous recombination (HR), such as MRE11, RAD50, NBS1, RAD51, BRCA1, BRCA2, and BRCC3. Downregulation of HR genes correlated with increased levels of their predicted microRNAs (miRNAs), miR-183 (predicted target RAD50) and miR-24 (predicted target BRCA1), suggested that pomegranate extract might regulate miRNAs involved in DNA repair processes. In a study, Adams demonstrated that Kinetic analysis of Urolithin B (UB) derived from ellagic acid (by gut microflora) showed mixed inhibition, suggesting more than one inhibitory mechanism. Proliferation assays also determined that UB significantly inhibited testosterone-induced MCF-7aro cell proliferation. The remaining test compounds also exhibited anti-proliferative activity, but to a lesser degree when compared to UB.

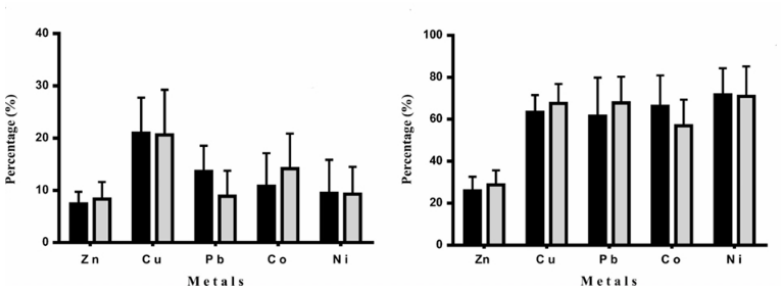

Figure 1: Cell viability (MTT), Zinc and Iron measurement of evaluating groups in timeframe of 24, 48 and 72 hours after treatment.

Zinc samples

In groups, the only increase had been observed in control in timeframe 48 hours to 24 hours as 3.6%. Also, the highest rate of decrease was shown in group A in 72 hours to 48 hours in amount of 13.8%, and on the other hand, the lowest rate of decreases observed in 2 groups of D and E, both in timeframes 72 hours to 48 hours as 4% shown in Figure 1. In our study, Zinc samples, all showed a decrease in all testing groups. The demonstrated significant differences on normal cell line survival of 108.7% at 10 μM supplemented medium was seen. Cytotoxicity was seen at 80 μM of 89.78% and 86.46% in MDA-MB-231 and U-87-MG Cell lines, respectively. In a study by Lu demonstrated that clioquinol combined with zinc markedly increased the radio sensitivity of HeLa and MCF-7 cells in low toxic concentrations and resulted in a post-irradiation decrease in G2 phase arrest and an increase in apoptosis.

Iron samples

The highest rate of decrease was observed in group E between timeframes of 48 hours to 24 hours as 44.3%, respectively. Also, the most increase was observed in group Control in timeframes 48 hours to 24 hours as 46.2%, respectively shown in Figure 1. In control group, there was an increasing and after 48 hours decreasing that may because of the cell's growth. In tests, in group B and group C approximately there are the same in amount and tolerance of soluble iron. In combination, the best one in terms of starting and consuming amount was observed in group E. In this group amount of 24 hours test is more than others and timeframes of 48 hours and 72 hours there are more consuming than other groups. In a study by Jiang and Elliott, revealed that increased iron in cancer cells and their microenvironment protects cancer cells from natural killer cell cytolysis by antagonizing NO- and TNFα-associated cytotoxicity and by up-regulation of ferritin expression in breast cancer cells.

For conclusion of all the tests, in control groups, we had cell growth and the amount of Iron increasing and Zinc decreasing; But for the other tests, in terms of non-combinations all the tests were descending (Except viability for ETE 100%). It means that when the single herbal, treating the cancer was successful; but for combinations, we had some confusing results those may because of the non-matching herbal dosages. We had unexpected viability in group F and also. We had very occasional viability in group D. So, that was suggested to test combination with other dosages to match these two herbs. In a study by Kumar in, the combination of Curcumin and Ellagic acid at various concentrations showed better anticancer properties than either of the drug when used alone as evidenced by MTT assay. Besides this, Curcumin and Ellagic acid also restore p53, induce ROS formation and DNA damage. Mechanistic study further indicated that Curcumin and Ellagic acid show anti-HPV activity as evidenced by decrease in the HPV E6 oncoprotein on HeLa cells. In other research program, the results verified the dose dependent anti-mutagenic effect of ellagic acid, curcumin and their combinations in both tests. The significantly increased effect of some combinations on the mutagenicity of indirect mutagens in the Ames test and on the direct mutagenicity of MNU in the micronucleus test, as compared with effect of ellagic acid or curcumin used separately, was also ascertained [21,22].

The dominant effective material of turmeric is Curcumin with chemical formula of C21H20O and the dominant material of pomegranate parenchyma is Punicalagin with chemical formula of C48H28O30. Both of them has phenolic ring with high affinity. So, it may need a catalyzer for best effect. So, more study with combination with other dosages to matching the herbals are suggested.

Conclusion

In conclusion of all the tests, in control groups, we had cell growth and the amount of Iron increasing and Zinc decreasing. But for the other tests, in terms of non-combinations all the tests are descending (Except viability for group B). The dominant effective material of turmeric is Curcumin have phenolic ring with high affinity. So, it may need a catalyser for best effect. So, more study with combination with other dosages to matching the herbals are suggested.

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Declarations

The authors declare that there is no conflict of interest regarding this article.

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